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Long term storage of cherry laurel (*Prunus laurocerasus* L.) and sweet cherry (*Prunus avium* L.) pollens

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Abstract

Pollen is an important research material for the biological and morphological studies and plays a magic role in reproduction process of fruit species. Storage of pollen is very important stage of breeding studies. In this research, Pollens of 6 cherry laurel types and 2 sweet cherry cultivars was evaluated for germination following storage two years at room temperatures, in refrigerator (4 ºC) and in deep-freezer (–25 ºC). Germination was obtained in medium containing 0.5% agar, 15% sucrose and 5 ppm boric acid and tests were made at the end of 90, 180, 270 and 730 days. Cherry laurel pollens stored at room temperatures lost the germination ability faster than pollens stored at the 4 or -25 ºC but nearly half of them still showed germination after 90 days. Among the cherry laurel types, type 36 gave the highest respect to storage with the best performance after 730 days storage period at -25 ºC (59.62%) while type 25 pollens germination percentage was only 21.86%. Sweet cherry cultivars were more sensitive to storage conditions and pollen viability was completely lost for 0900 Ziraat cultivar after 90 days storage at the room temperature while Prime Giant germination percentage was only 2.05%. Germination of 0900 Ziraat and Prime Giant pollens stored at -25 ºC was 21.54% and 33.50% respectively, at the end of 730 days storage period. These results provided preliminary information for the storage conditions of pollens and pollination in breeding programs of both of cherry laurel and sweet cherry species.

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Introduction

Pollen is a simple plant cell and pollen tube formation is a good and simple model of growth and development (Taylor and Hepler, 1997; Mautinho *et al.*, 2001). Thus, pollen germination and growth of pollen tubes are important research materials for morphological, physiological, biotechnological, ecological, evolutional, biochemical and molecular biological studies (Ottavio, 1992; Wang *et al.*, 2004; Radicevic *et al.*, 2013). On the other hand, pollen morphology has an importance in clarifying the classification of many plants (Kaya *et al.*, 2013; Radicevic *et al.*, 2013).

Pollen quality is one of the key factors of reproduction process in different fruit species and varies among cultivars within a species (Eti *et al.*, 1995; Stosser *et al.*, 1996; Pırlak, 2002). Pollination is a very important in regular and sufficient fruit production and pollination with high quality pollens and fertilization are most important factors affecting fruit setting (Sütyemez, 2011; Sharafi and Bahmani, 2011). Pollen handling techniques, storage of pollens and suitable methods for testing viability and germination are an important part of these procedures (Farmer and Hall, 1975; Pırlak and Bolat, 1999; Dafni and Firmage, 2000). If the male parents flowers open first require the storage of pollen for a short time only, but if the female parent flowers are first, we think about storage of pollens for a year until next flowering season (Howard, 1958). Pollen collected from early blooming types and stored at sub-zero temperatures for very long period without any appreciable loss of viability can be used along the whole blooming season for hybridization program by fruit breeders (Bhat *et al.*, 2012). Factors to be considered which should influence stored-pollen longevity are environmental conditions such as temperature and humidity, maturity of pollen, handling time and methodology and packaging. Pollen viability may decrease depending upon the storage conditions in time (Griggs *et al.*, 1953; Farmer and Hall, 1975; Bolat and Güleryüz, 1994; Alburquerque *et al.*, 2007; Sharafi and Bahmani 2011; Bhat *et al.*, 2012).

Cherry laurel, a small cherry in red colour at maturity stage, is one of the native fruit in the region of Black Sea, Southwestern Asia and Southeastern Europe. Breeding studies began with selection of the superior types from natural populations and some morphological, phenological, pomological and biochemical characteristics were determined; furthermore, molecular biological studies and cultivation possibilities has started to work (Beyhan, 2010; Halilova and Ercişli, 2010; Sulusoglu, 2011; Macit and Demirsoy, 2012; Hajyzadeh *et al.*, 2013). It is mostly consumed as a fresh or dried fruit and also used as an ornamental plant in Europe and United States. Cherry laurel has an increasing popularity so that, propagation possibilities with traditional methods and micropropagation approaches was studied for the future breeding studies too [Ponchia, 1991; Posta, 2009; Riberio *et al.*, 2010; Sulusoglu and Cavusoglu, 2010; Sulusoglu, 2012; Sulusoglu and Cavusoglu, 2013a; Sulusoglu and Cavusoglu, 2014). On the other hand, a study was contributed to develop sterile cherry laurel cultivars as a part of a breeding program (Contreras and Meneghelli, 2013). As breeding of the cherry laurel fruit species intensifies, there will be an increasing need for effective controlled crossing procedures. Flowering dates of cherry laurel types change from the third week of March to third week of April for the cherry laurel types (Sulusoglu, 2011) and bloom times sometimes do not overlap between cherry laurel cultivars. These differences require that pollen is collected and stored for a special time. Pollen germination of cherry laurel types was discussed in the earlier study (Sulusoglu and Cavusoglu, 2013b) but protocols for pollen storage were not described. In the highlight of the listed using areas, storage of pollen is very important stage for the genetics and breeding studies and also for the gene conservation.

The other specie 'sweet cherry' that studied here is highly appreciated by consumers due to its excellent quality. Turkey is the biggest sweet cherry producer country in the world and the most common cultivar grown is '0900 Ziraat' (Kaynaş *et al.*, 2008).

Sweet cherry is a well suited specie for pollen

competition studies with its high pollen-ovule ratio and pollen germination and storage work has been performed (Hormaza and Herrero, 1996) and most of sweet cherry cultivars are self-unfruitful as well as cross-unfruitful (Crane and Brown, 1937; Eti *et al.*, 1995; Hormaza and Herrero 1999; Pırlak 2002; Tosun and Koyuncu 2007; Radicevic *et al.*, 2013). The success with Napoleon cherry cultivar pollen germination after storage for 408 days in a home freezer at approximately -20 ºC (Griggs *et al.*, 1953) and the early work of Alburquerque (2007) that sweet cherry pollen stored at -20 ºC for about a year or more, is advantageous for the next studies. Such methodology is useful to efficiently plant hybridizations between cultivars flowering very separately in time. There are some studies on pollen germination of 0900 Ziraat but Prime Giant is a new and very favorable sweet cherry cultivar and could not be reached any study on pollen germination and characteristics of Prime Giant cultivar. On the other hand, long-term pollen storage of these cultivars has not been obtained according to our literature review. The objective of this work was to determine the detailed pollen storage management protocol of 6 cherry laurel types and 2 sweet cherry cultivars (Prime Giant and 0900 Ziraat) pollens stored at different temperature conditions.

Materials and methods

Materials

Pollens of 6 cherry laurel types (types 16, 25, 34, 36, 37 and 39) which grown in naturally in Kocaeli City, North-Western part of Turkey that involved in the selection study of the superior cherry laurel (*P. laurocerasus* L.) types (Sulusoglu, 2011), and 2 commercially important sweet cherry cultivars (*P.avium* L.), 0900 Ziraat and Prime Giant was grown in the garden of Kocaeli University Arslanbey Vocational School were used in this study.

Methods

The study was conducted over 2011-2014 years and unopened flowers were collected in white balloon stage in March and April of 2011 and 2012 years from all sides of the trees and carried to the laboratory immediately. After petals and sepals were separated, anthers isolated from flower buds and placed on a black paper under an incandescent lamp on a table overnight. After desiccation, pollens were placed in small glass bottles; lids were closed and wrapped with stretch film carefully. Glasses were placed in a lidded plastic container that including silica gel at the bottom and stored at room temperature, in the refrigerator (4 ºC) or in the deep-freezer (-25 ºC). Bottles have prepared separately for each treatment to avoid the open the cover of glass more one times.

Pollen was removed from storage at the end of 90, 180, 270 and 730 days storage time for germination tests and dusted onto Petri dishes with 20 ml of medium containing 0.5% agar, 15% sucrose and 5 ppm boric acid (H_3BO_3) (Sulusoglu and Cavusoglu, 2013b). Petri dishes were incubated in an oven, dark condition for 24 hours at 22 ºC. Lids were closed on the Petri dishes and insured high humidity.

At the end of a 24 hours incubation period, germination percentages were determined by counting each replicate. An optical microscope (10x ocular and 10x or 40x objective) was used to ascertain the number of germinated and non-germinated pollen grains and pollen grains were considered as germinated when the length of the pollen tube exceeded its diameter (Brown, 1958). The experiment were designed as completely randomized plot design and for each treatment combination (pollen genotype, temperature and storage time), germination was recorded by counting in 5 different ocular fields for each Petri dishes and 3 Petri dishes were used as a replication. Germination percentages were transformed by arcsine root square and ANOVA analysis was carried out. Storage time and storage conditions interaction was analyzed separately for each cherry laurel types and sweet cherry cultivars. Germination rates of stored pollens were compared with the fresh (0 day) pollen germination rates as a control for each storage temperature. The differences among means were analyzed using the Duncan's multiple range test at $P<0.05$ significance.

Results

In the present investigation an attempt has been made to determine the germinability of cherry laurel and sweet cherry pollens to be stored at different temperature conditions. Pollen germination percentage was examined up to 730 days in room temperature, refrigerator (4 °C) and freezer (-25 °C).

Table 1. Germination value of cherry laurel Type 16 pollen grains in the different storage conditions during the storage period (%).

Storage period (days)	Room temperature*	Refrigerator (4 °C) *	Deep freezer (-25 °C) *
0	65.01 aA	65.01 abA	65.01 aA
90	32.15 bB	67.02 aA	72.03 aA
180	19.36 cB	50.89 bA	62.29 aA
270	0.00 dC	33.57 cB	56.98 abA
730	0.00 dB	0.00 dB	44.07 bA
Mean of storage conditions	22.70	43.30	60.08

Table 2. Germination of cherry laurel Type 25 pollens in the different storage conditions during the storage period (%).

Storage period (days)	Room temperature*	Refrigerator (4 °C) *	Deep freezer (-25 °C) *
0	71.33 aA	71.33 aA	71.33 aA
90	48.28 bA	62.83 aA	62.74 abA
180	37.32 bA	45.52 bA	51.56 bA
270	4.41 cB	24.64 cA	32.59 cA
730	0.00 dB	3.74 dB	21.86 cA
Mean of storage conditions	32.27	41.61	48.02

Effects of storage conditions on cherry laurel pollen germination

The germination percentages of the pollen grains of the 6 cherry laurel types are presented in Table 1, 2, 3, 4, 5 and 6. The germination rate on the first day of pollens was the recorded highest value for all the cherry laurel types and the highest germination percentage were obtained with type 36 fresh pollen grains (75.96%) (Table 4). Germination percentages of the room stored pollens decreased rapidly over the time and this decreasing was statistically important for all types of cherry laurel at the end of 90 days storage period when compared the first day germination values. Only a few pollens of 25, 36, 37 and 39 cherry laurel types could germinate after 210 days stored at the room conditions and germination percentages was highest for type 36 (12.83%). Germination power was completely lost at the end of two years storage period for all the cherry laurel types pollens stored at the room temperature.

Table 3. Germination value of cherry laurel Type 34 pollen grains in the different storage conditions during the storage period (%).

Storage period (days)	Room temperature*	Refrigerator (4 °C) *	Deep freezer (-25 °C) *
0	61.45 aA	61.45 aA	61.45 aA
90	20.63 bB	53.89 abA	57.27 aA
180	17.50 bB	49.63 bcA	55.70 aA
270	0.00 cB	41.97 cA	51.96 aA
730	0.00 cB	3.19 dB	34.65 bA
Mean of storage conditions	19.92	42.03	52.21

Refrigerator condition was a more suitable media for the protection of cherry laurel pollen grains viability but again, the loss of germination ability was a condition variable in time. Except type 39, there was not any statistical decreasing between the fresh pollen and 90 day stored pollen germination rates.

Germination results of pollen grains stored in the refrigerator was the least for the pollen grains of type 39 and highest for type 36 at the end of 180 days storage period (43.52% and 62.25% respectively) (Table 6 and 4). Even, type 16 and Type 37 pollens did not germinate at the end of two years storage period while others had only a few germinated pollens at the refrigerator conditions (Table 2, 4, 3 and 6).

There was no noticeable difference between the germination percentages of types 16, 34 and 36 pollens stored at -25 ºC temperature in 90, 180 or 270 days periods; statistical differences observed end of the second year (730 days) treatments for these types at -25 ºC storage temperatures (Table 1, 3 and 4).

Table 4. Germination value of cherry laurel Type 36 pollen grains in the different storage conditions during the storage period (%).

Storage period (days)	Room temperature*	Refrigerator (4 ºC) *	Deep freezer (-25 ºC) *
0	75.96 aA	75.96 aA	75.96 aA
90	31.83 bB	72.42 aA	76.42 aA
180	20.73 bcB	62.25 aA	73.00 abA
270	12.83 cB	61.54 aA	69.86 abA
730	0.00 dC	4.83 bB	59.62 bA
Mean of storage conditions	28.27	55.40	70.97

Table 5. Germination value of cherry laurel Type 37 pollen grains in the different storage conditions during the storage period (%).

Storage period (days)	Room temperature*	Refrigerator (4 ºC) *	Deep freezer (-25 ºC) *
0	66.30 aB	66.30 aA	66.30 aA
90	45.16 bB	69.41 aA	69.76 aA
180	32.43 bB	52.96 abA	53.42 abA
270	5.03 cC	30.19 bB	46.44 bA
730	0.00 dB	0.00 cB	47.29 bA
Mean of storage conditions	29.78	43.77	56.65

In spite of significant decreasing germination values of -25 ºC stored pollen after 730 days; it was an important result that only pollen grains stored at -25 ºC provided good viability retention and pollen tube development up to the two years for cherry laurel types (Figure 1 A, B, C, D, E and F). Cherry laurel type 36 gave the highest germination percentage (59.62%) at the end of two years storage time (730 days) inside the cherry laurel types pollens stored at -25 ºC (Table 4). Meanwhile, storage pollens in the refrigerator or deep freezer had significantly higher germination percentages than pollens stored at the room temperature but not significantly higher than each other until end of the 270 days storage period for type 16, 37 and 39 (Table 1, 5 and 6); and until end of the 730 days storage period for types 25, 34 and 36 (Table 2, 3 and 4).

Effects of storage conditions on sweet cherry pollen germination

Cold storage significantly extended the storage time for the sweet cherry cultivars pollen and this effect was more clear results according to the cherry laurel. The data concerning germination percentage of pollen grains of the sweet cherry cultivars were shown in Table 7 and 8 and data presented in these tables showed that stored pollen grains both of sweet cherry cultivars lost germination ability dramatically at the room temperatures. Among the sweet cherry cultivars, Prime Giant gave the highest germination value as a 63.74% in the first day experiments and at the end of 90 days room storage, still had a few germinated pollens too (Figure 2 A and B). The reduction in germination capacity was clearer for sweet cherry cultivar 0900 Ziraat. Under refrigerator

conditions, sweet cherry pollens lost their longevity gradually by the time; after 90 days storage period 0900 Ziraat cultivar germination losses were observed in almost half while Prime Giant pollen germination power was nearly the two-thirds of the first day germination values (Figure 2 C). While none of pollen grains of 0900 Ziraat germinated after two years storage in the refrigerator, only 1.67% percentage germination value was recorded for the Prime Giant cultivar. Only pollen grains stored at -25 ºC provided remarkable viability retention until end of two years storage period for both of sweet cherry cultivars (Figure 2 D and E) and Prime Giant again gave the highest germination percentages (33.50%) (Table 7 and 8).

Table 6. Germination value of cherry laurel Type 39 pollen grains in the different storage conditions during the storage period (%).

Storage period (days)	Room temperature*	Refrigerator (4 ºC) *	Deep freezer (-25 ºC) *
0	73.53 aA	73.53 aA	73.53 aA
90	49.78 bB	57.00 bAB	68.36 aA
180	20.55 cB	43.52 bA	52.12 bA
270	0.98 dC	27.90 cB	43.11 bcA
730	0.00 dB	0.65 dB	35.68 cA
Mean of storage conditions	28.97	40.52	54.56

Discussion

Pollen performance means includes pollen germination, pollen tube growth rate and pollen competition which is an important key in the fertilization of flowering plants (Hedhly *et al.*, 2005). Genetic material and storage conditions of pollens effects pollen longevity (Griggs *et al.*, 1953; Dane *et al.*, 2004; Mert, 2009; Hedhly *et al.*, 2005).

Table 7. Germination value of sweet cherry cultivar 0900 Ziraat pollen grains in the different storage conditions during the storage period (%).

Storage period (days)	Room temperature*	Refrigerator (4 ºC)*	Deep freezer (-25 ºC)*
0	53.25 aA	53.25 aA	53.25 aA
90	0.00 bC	19.19 bB	37.38 bA
180	0.00 bC	14.84 bB	30.78 bcA
270	0.00 bC	5.70 cB	24.16 cA
730	0.00 bB	0.00 dB	21.54 cA
Mean of storage conditions	10.65	18.60	33.42

Table 8. Germination value of sweet cherry cultivar Prime Giant pollen grains in the different storage conditions during the storage period (%).

Storage period (days)	Room temperature*	Refrigerator (4 ºC)*	Deep freezer (-25 ºC)*
0	63.74 aA	63.74 aA	63.74 aA
90	2.05 bB	42.91 bA	57.81 aA
180	0.00 bB	27.54 cA	36.75 bA
270	0.00 bB	24.75 cA	35.13 bA
730	0.00 bB	1.67 dB	33.50 bA
Mean of storage conditions	10.65	18.60	33.42

*Values in the same column (storage condition) with different lower-case letters and values in the same row (storage period) with different capital letters are significantly different (P<05).

In this work, significant differences were recorded in pollen germination performance inside the cherry laurel types at each temperature storage conditions as mentioned in other studies that cherry laurel types germination capacity decreased over the storage time

and especially sweet cherry cultivars pollens completely lost germination ability after 90 days storage period at the room temperature. Refrigerated pollens of cherry laurel and sweet cherry was conveniently stored for short or mid periods while frozen pollen stored for longer time. The same results found by Alburquerque *et al.*, (2007) for sweet cherry cultivars that pollen viability was completely lost in of most cultivars after only 60 days of storage at 4 ºC; however percentages of the germinated pollen were not different from the control after one year storage at -20 ºC. Again was listed in the earlier reports that, most of fruit species could protect pollen germination capacity with slight losses in time when stored at sub-zero temperature (Griggs *et al.*, 1953; Farmer and Hall, 1975; Martinez and Gomez, 2002; Alburquerque, 2007; Perveen *et al.*, 2007). On the other hand, date palm pollen longevity is increased by cryopreservation of pollens (Ateyyeh, 2012). Theoretically, storing pollen in liquid nitrogen for many years is possible, because biological activity is stopped (Withers, 1991) as in our study, freezing process was reduced germination losses.

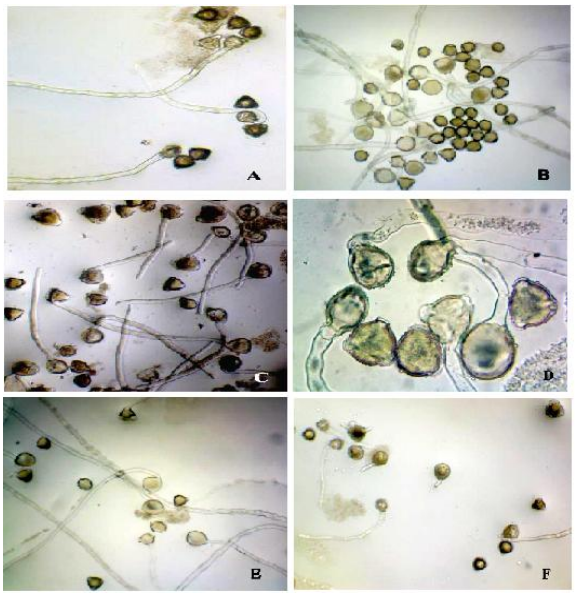

Fig. 1. Pollen germination of cherry laurel types after 730 days storage at the -25 ºC.

A)Type 16;

B) Type 25;

C) Type 34

D) Type 36

E) Type 37;

F) Type 39.

If the reduction in pollen viability is severe depending on the storage conditions, like as stored pollen of cherry laurel types and sweet cherries at the room temperature, germination could not occur even in excellent conditions. Under room temperature storage conditions, cherry laurel types and sweet cherry cultivars had lost their longevity gradually by the time; after 270 days storage period the pollen did not germinate or germination rate was very low. This effect was even more explicit in the germination of sweet cherry pollens and in this respect the present work results are generally in agreement with the findings of other investigators (Duffield, 1954; Sharafi and Bahmani, 2011; Ateyyeh, 2012.).

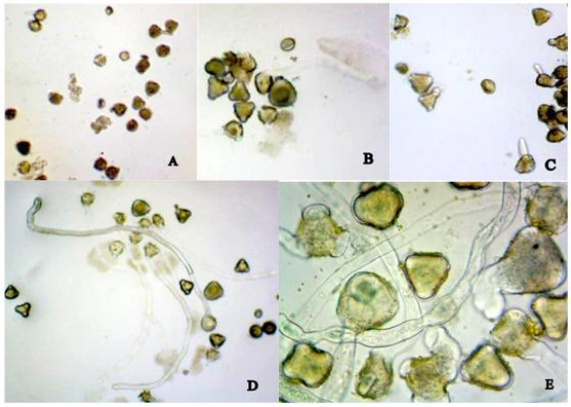

Fig. 2. Pollen germination of sweet cherry cultivars

A) Non-germinated pollen of 0900 Ziraat after 90 days storage at the room temperature

B) Pollen germination of Prime Giant after 90 days storage at room temperature

C) Pollen germination of Prime Giant after 730 days storage at 4 ºC.

D) Pollen germination of 0900 Ziraat after 730 days storage at -25 ºC.

E) Pollen germination of Prime Giant after 730 days storage at -25 ºC.

As has been frequently observed in the other species (Ateyyeh, 2012; Bhat *et al.*, 2012), there were major variations in germination capacity among the pollens of different types of cherry laurel and sweet cherry cultivars after stored at different storage temperatures. According to our results, the germination losses were faster for the sweet cherry cultivars and 0900 Ziraat was the sensitive one.

Similar results were obtained by Sharafi and Bahmani (2011), that genotype significantly affected the pollen storage of the sweet cherries cultivars. The increasing importance and potential uses of cherry laurel in many parts of the world, highlights the need forresearch into their breeding systems and in the development of improvement methods. This research has shown that genotype improvement is an achievable goal, to which pollen storage techniques can contribute in a major way. Not only storage the pollen for a long period, but also to know the effectiveness in pollination is the important criteria for the practical studies and in the next studies, in vivo fertilization test should be organize to improve the use of them as a pollinizer in the breeding programs.

Reducing moisture content and storage pollen in air-dried system increase the longevity of desiccation tolerant pollen (Roberts, 1975; Hong *et al.*, 1999). To use sealed glass bottles and saved them in lidded plastic box including silica gel, helped to protect the humidity condition in this study but in the next studies to determine the moisture content of pollen and supply humidity-controlled condition should be effect the germination rates.

0900 Ziraat is a favorable cherry cultivar and fresh pollen germination percentage was found 36.25% by Tosun and Koyuncu, (2007). In our study, the fresh pollen germination percentage was higher (53.25%) and 33.42% of pollens protected germination capacity with the mean value of 730 days storage period. On the other hand, 64.74% germination percentage is the first data for Prime Giant pollens as well as we known.

In conclusion, this was the first study for storage of cherry laurel pollens and pollen germination rates following storage varied with cherry laurel types. Fresh pollen should be used rather than stored whenever possible, but sometime storage of the pollen is a necessity for the controlled pollination program. For shorter storage periods of cherry laurel pollen grains, to keep the sealed vials at 4 ºC temperatures in the refrigerator adequately protect the viability and allow using in the fertilization studies within the same season. Although, cherry laurel pollens were more long-lived than sweet cherry pollens and were less sensitive to temperature environment in the storage, the principal conclusion which can be drawn from this study is that both of species were affected from the storage temperatures. Only storage at lower temperatures (as -25 ºC) provided remarkable germination retention for sweet cherry cultivars pollens. As a result of, we could say that storage of sealed vials at -25 ºC proved to be a simply effective way of storing pollen grains by protecting the near to half of them up to two years and these results will support the studies basic aspects of pollen physiology and biochemistry need exploration in future and will serve to biological and genetic studies without time limitation.

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